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Report

A Complex between FAK, RACK1, and PDE4D5 Controls Spreading Initiation and Cancer Cell Polarity

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Summary

A fundamental question in cell biology concerns how cells respond to their environment by polarizing after sensing directional cues. This requires the differential localization of protein complexes in cells, and it is important to identify and understand how these complexes function. Here we describe a novel "direction-sensing" pathway that links the integrin effector focal adhesion kinase (FAK [1–7]), the molecular scaffold protein RACK1 [8–10], and activity of one of its client proteins, PDE4D5, a cAMP-degrading phosphodiesterase. The complex is recruited to nascent adhesions and promotes cell polarity. We identify FAK FERM domain residues whose mutation impairs RACK1 binding. When re-expressed in cancer cells in which endogenous *fak* is deleted by Cre-lox-mediated recombination, the RACK1-binding-impaired FAK mutant protein does not support formation of nascent actin adhesion structures as cells spread. These cancer cells, like FAK-deficient cells, cannot undergo directional responses, including wound-induced polarization or chemotactic invasion into three-dimensional matrix gels. We show that RACK1 serves as the molecular bridge linking FAK to the recruitment of PDE4D5. FAK/RACK1/PDE4D5 is a novel 'direction-sensing' complex that acts to recruit specific components of the cAMP second-messenger system to nascent integrin adhesions and to the leading edge of polarizing cells.

Results and Discussion

FAK and RACK1 Form a Complex in Squamous Cancer Cells and Colocalize at Nascent Adhesions

We previously showed that focal adhesion kinase (FAK) was present, and colocalized with RACK1, at nascent protrusive structures that form as mouse embryonic fibroblasts (MEFs) spread on extracellular matrix (ECM) [11]. Here we have derived cells from chemically induced squamous cell carcinomas (SCCs) in mice that expressed a *floxed* form of FAK under the control of skin-specific Cre recombinase via the keratin-14 promoter [12].

FAK deficiency (FAK-null) in SCCs (as well as in MEFs) caused severe spreading defects, as judged by a lack of newly formed protrusive structures after 30 min on fibronectin (FN) (Figure 1A). FAK and RACK1 colocalized at nascent structures in SCCs; however, RACK1 was largely cytoplasmic in FAK-null cells (Figure 1A). We showed that these structures were in close proximity to the cell-substratum interface by using total internal reflection fluorescence (TIRF) microscopy (Figure 1B). The structures did not form properly when FAK-null cells first contacted the FN-coated substratum (Figure 1B). We also noted that there were small "spots" of colocalization between FAK and RACK1 at the periphery of fully adherent cells. These spots of colocalization were best seen by TIRF microscopy (Figure 1C). These data indicate that FAK and RACK1 were also selectively colocalized in motile adherent cells, most likely at transient nascent contacts close to the cell-substratum interface, but not at more mature focal adhesions. Thus, the RACK1-containing structures are early cell-ECM contacts that can be distinguished from mature focal adhesions by the presence of RACK1 (similar structures have been described before [13]). The complete absence of FAK after treatment with 4-OHT was confirmed (Figure S1, available online), and we used these cells to re-express mutant FAK proteins to around 1.5–2 times endogenous FAK levels.

FAK formed a complex with endogenous RACK1 in suspended SCC cells, and this persisted after plating on FN (Figure 1D). We noted that FAK-Tyr-397 was constitutively phosphorylated on the integrin-induced autophosphoryation site, even when placed in suspension, showing that FAK activity was deregulated, and uncoupled from cell adhesion, in these cancer cells (Figure 1D, right panel). We showed that the FAK/RACK1 complex was independent of FAK-Y397 phosphorylation and that, as expected, the FAK/Src complex was abolished in FAK-Y397F-expressing cells (Figure 1E). We confirmed specificity of the IP by use of nonimmune IgG (Figure 1E, right panel). Thus, FAK promotes nascent adhesion contacts, and FAK binds the scaffold protein RACK1. The two proteins colocalize at nascent contacts close to the cell-substratum interface of spreading cells.

FAK-RACK1 Binding Involves the FAK FERM Domain and Controls RACK1 Localization and Spreading Initiation

A scanning peptide array of full-length FAK was generated as a library of overlapping 25-mer peptides; each sequence was displaced 5 amino acids toward the C terminus, as in 1–25, 6–30 etc. This immobilized peptide array was probed with either purified GST or a purified GST-RACK1 fusion protein and used, in conjunction with alanine scanning mutagenesis, for identifying amino acids whose mutation might interfere with the FAK-RACK1 interaction, thus guiding the generation of a putative effector mutant (described in detail in the legend to Figure S2A). The potential binding regions predicted by this approach were in the FAK FERM domain, identifying RACK1 as a novel FERM-interacting protein.

We made two putative interfering mutations in full-length FAK and expressed the mutants in FAK-deficient SCCs. One of these, FAK-E139A,D140A, greatly impaired binding of FAK to RACK1 (Figure 2A, left panel). The other mutant,

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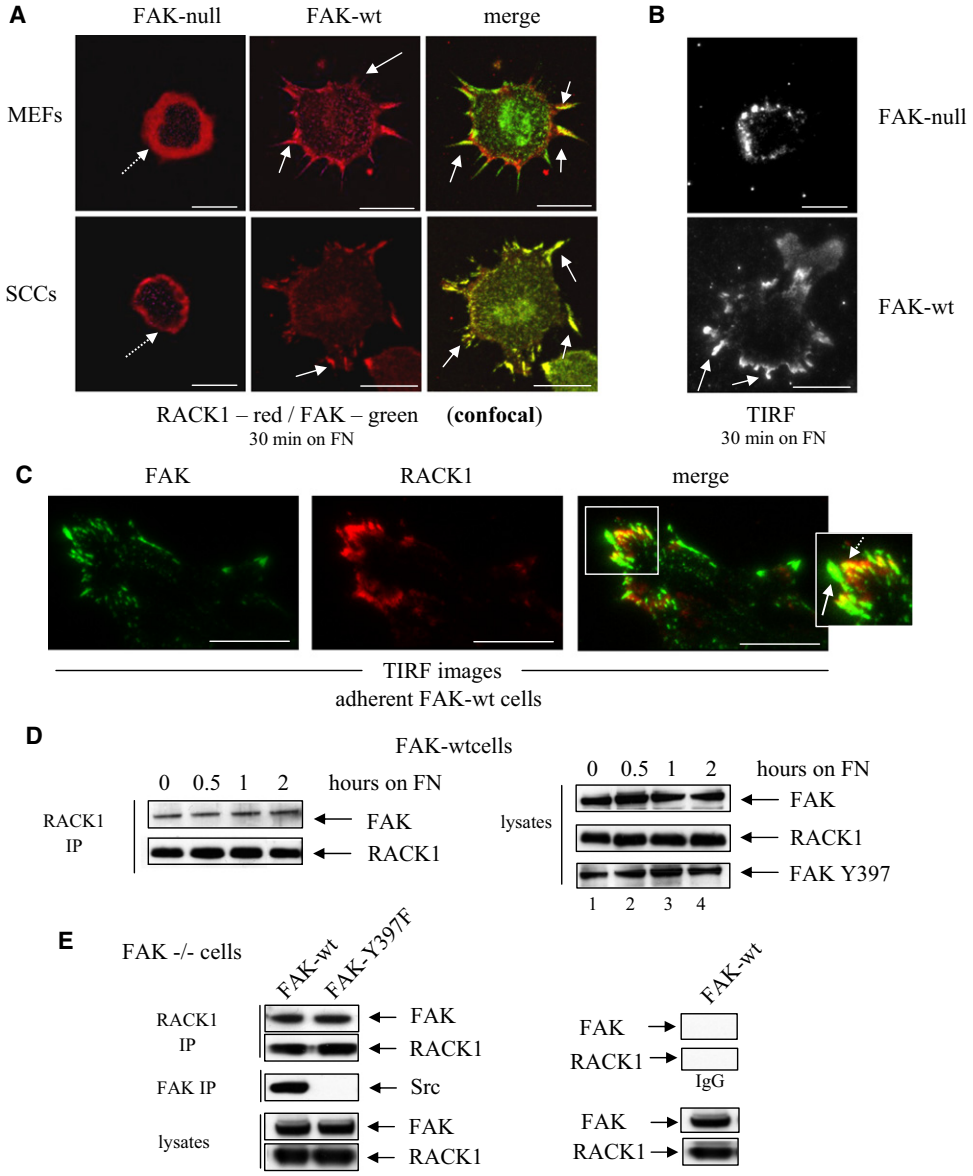

Figure 1. FAK and RACK1 Form a Complex in Squamous Cancer Cells and Colocalize at Nascent Adhesions

(A) FAK-null or FAK-wt SCCs and MEFs were suspended in PBS for 1 hr and then plated on FN (10 μg/ml) for 30 min. Cells were fixed and stained with anti-RACK1 (red) and anti-FAK (green) and visualized with confocal miocroscopy. Merged images are shown (right panels). Solid arrows indicate colocalization , and broken arrows indicate cytoplasmic RACK1. SCC cells plated on FN were stained with (B) anti-RACK1 or (C) anti-RACK1 (red) and anti-FAK (green), then imaged via TIRF. Merged and zoomed images are shown. A solid arrow shows mature focal adhesion, and a broken arrow indicates small nascent adhesion. (D) FAK-null SCCs re-expressing FAK-wt were suspended and then plated on FN. RACK1 was immunoprecipitated (IP), and immunoblotting was carried out with anti-FAK, anti-RACK1, and anti-FAK Y397 as probes. (E) RACK1 was immunoprecipitated from FAK-wt or FAK-Y397F cells. Immunoblotting was carried out with anti-FAK and anti-RACK1 as probes. FAK was also immunoprecipitated from these cells, and immunoblotting was carried out with anti-FAK and anti-Src as probes. Control IP is shown for non-immune IgG (right panel), with anti-FAK and anti-RACK1 as probes. Scale bars represent 20 μm.

FAK-E237A,E238A, did not (sometimes such false positives arise in binding arrays and inhibition of binding is not replicated in the context of the full-length protein in cells). FAK-E139A,D140A did not affect the association of FAK with Arp3, which bound to a different region of the FAK-FERM domain (Figure S2B [11]). Further characterization of this RACK1-binding-deficient FAK mutant showed that it was tyrosine phosphorylated at Y397 and was associated with phospho-Src and paxillin (Figures S2C and S2D). Although RACK1 binds Src [14], we did not find that RACK1 association with Src was altered in cells expressing the FAK-E139,D140A mutant (Figure S2E).

Disruption of the FAK/RACK1 complex in adherent cells impaired the ability of RACK1 to locate to protrusive structures at cell edges, and the cells were poorly spread. They appeared either round or triangular, and RACK1 was cytoplasmic (Figure 2B and Figure 2C). In FAK-wt- and FAK-E237A:E238A-expressing cells, RACK1 was concentrated in the vicinity of membrane protrusions, and there were nonstaining regions between the protrusions and the nucleus, as shown by digital

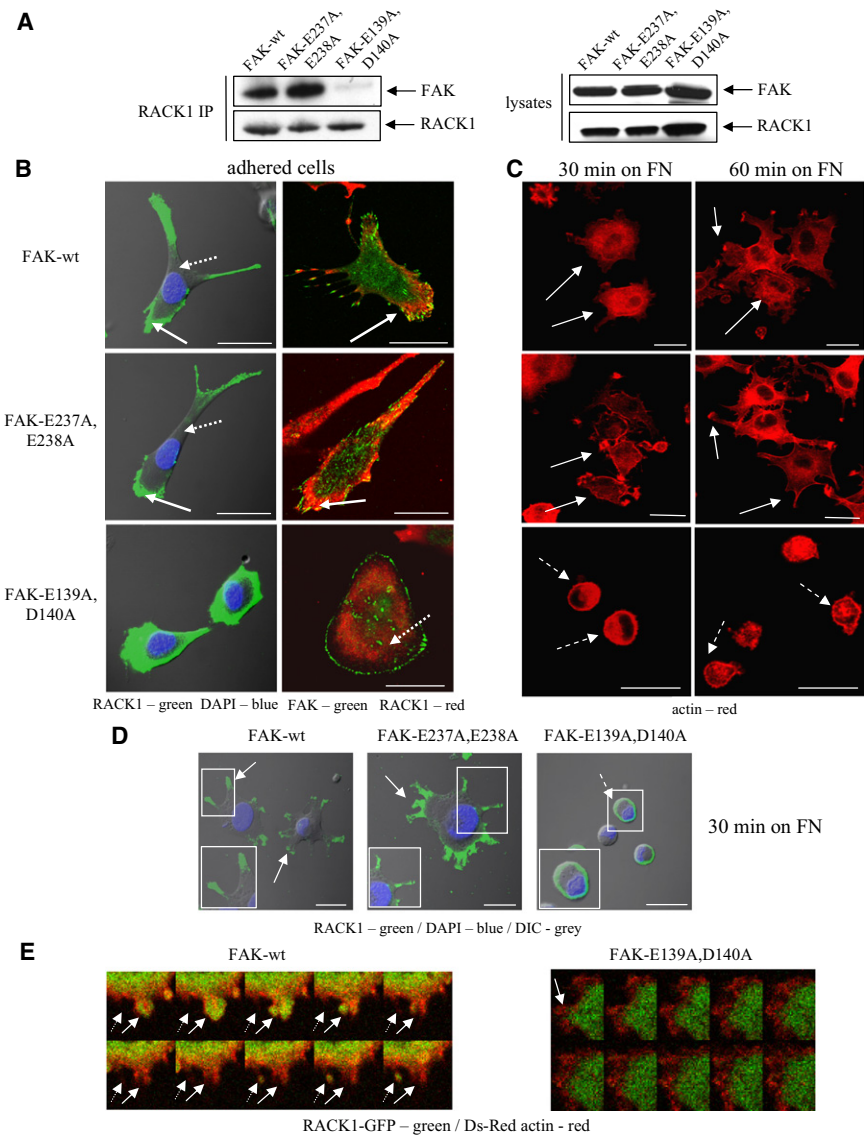

Figure 2. FAK-RACK1 Binding Involves the FAK FERM Domain and Controls RACK1 Localization and Spreading Initiation

(A) RACK1 was immunoprecipitated from FAK-wt, FAK-E237A,E238A, or FAK-E139A,D140A cells. Immunoblotting was then carried out with anti-FAK and anti-RACK1 as probes.

(B) Adherent SCC cells were fixed and stained with anti-RACK1 (green) and DAPI (blue) (left panels) or with anti-RACK1 (red) and anti-FAK (green) (right panels). Solid arrows indicate peripheral RACK1, and broken arrows indicate cytoplasm (left panels) or cytoplasmic RACK1 (right panels).

(C) FAK-wt, FAK-E237A,E238A, or FAK-E139A, D140A cells were suspended, plated on FN, and then fixed and stained with TRITC phalloidin (red). Solid arrows represent spreading cells, and broken arrows indicate rounded cells.

(D) Cells were suspended, plated on FN, fixed and stained with anti-RACK1 (green) and DAPI (blue), and imaged with DIC. Solid arrows indicate the formation of protrusive structures, and broken arrows indicate a lack of such structures. Scale bars represent 20 μm.

(E) FAK-wt (left panels) or FAK-E139A,D140A (right panels) cells expressing Ds-Red actin and RACK1-GFP were plated on FN and imaged in real time every minute for 20 min (Movie S1 and Movie S2). Still series from zoomed images are shown. In the left series, a solid arrow shows protrusion in which RACK1-GFP moves back to the cytoplasm while actin remains, and a broken arrow shows a new actin-based adhesion that forms later and at which RACK1-GFP is present. In the right series, an arrow shows dynamic peripheral actin ruffles that are devoid of RACK1-GFP and do not stabilize.

image contrast (DIC) (Figure 2B, solid arrows). RACK1 did not colocate with FAK at the periphery of FAK-E139A,D140A-expressing cells, and these cells had less obvious directionality (cells were triangular in shape and had smooth edges; Figure 2B, lower right panel). Costaining of FAK with paxillin at the cell periphery is shown in Figure S2D, right panel. Actin staining in cells expressing FAK-E139A,D140A revealed that these were poorly spread at 30 and 60 min after plating on FN, and assembly of protrusive actin structures was reduced (Figure 2C), reminiscent of FAK-deficient cells. Quantification of cells with actin protrusions is shown (Figure S2F). In spreading cells, the concentration of cellular RACK1 in the vicinity of protrusions was not seen in cells expressing the FAK-E139A,D140A mutant, which remained rounded (Figure 2D).

We next generated time-lapse movies of cells expressing Ds-red actin and RACK1-GFP as they contacted FN-coated substrata (Movie S1 and Movie S2). Still images from these are shown (Figure 2E). In the case of FAK-wt-expressing cells, dynamic actin-based protrusive "blebbing" structures were evident, and RACK1 entered and left these over time. In stark contrast, although there was actin ruffling activity at the edges of cells expressing the FAK-E139A, D140A mutant, RACK1-GFP did not "visit" peripheral actin structures, and protrusions did not form or persist (Figure 2E). These data are consistent with RACK1's "visiting" peripheral actin structures transiently in a FAK-dependent manner as they assemble and suggest that the FAK/RACK1 complex delivers a signal that promotes stabilization of dynamic "first-contact" adhesions.

FAK-RACK1 Binding Is Needed for Wound-Induced Cell Polarization and Chemotactic Cancer Cell Invasion

We next examined the ability of FAK-E139A,D140A cells to polarize toward a wound created in a confluent monolayer by using an assay that visualized the orientation of Golgi relative to the nucleus [15]. FAK-deficient (FAK-null) cells and cells re-expressing FAK-E139A:D140A were unable to polarize toward the wound, demonstrating either an inability to sense the space created by the wound or an inability to turn toward it (Figure 3A). In parental SCC cells and FAK-null cells re-expressing wild-type FAK (FAK-wt), endogenous RACK1 localized along the leading edge of cells at the wound front (Figure S3A, solid arrows). In contrast, RACK1 did not efficiently localize to the polarizing edges of FAK-null or FAK-E139A,D140A cells, and the cells were disorganized (Figure S3A, broken arrows).

Next, cells were seeded onto transwell filters and allowed to invade through Matrigel toward a chemotactic stimulus (in this

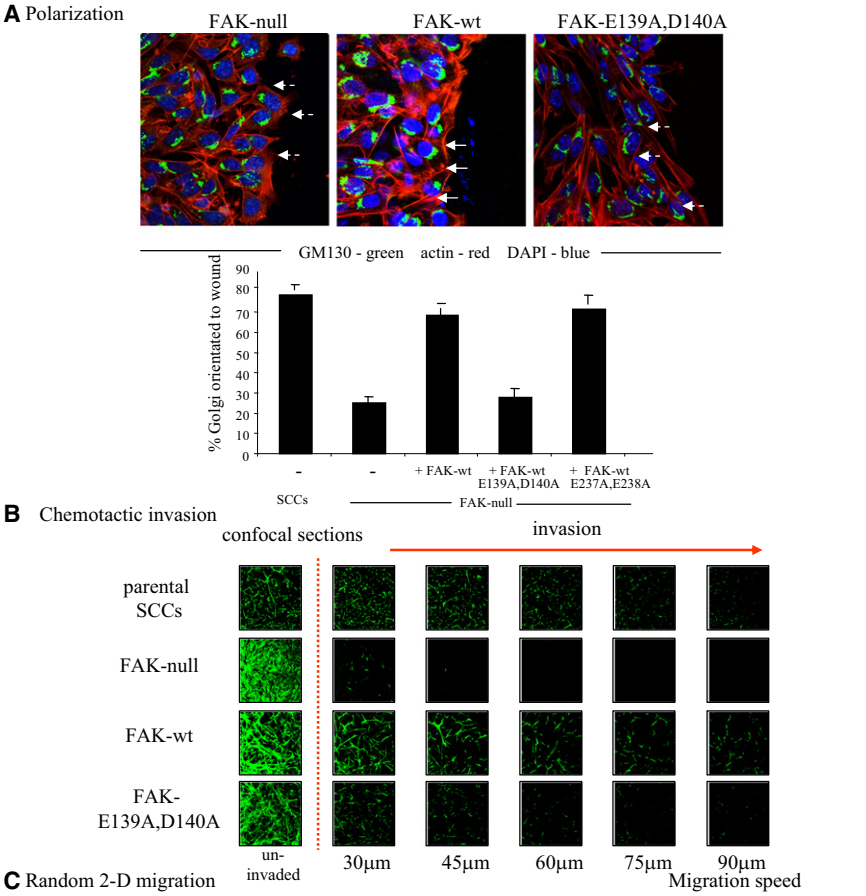

Figure 3. FAK-RACK1 Binding Is Needed for Wound-Induced Cell Polarization and Chemotactic Cancer Cell Invasion

(A) FAK-wt, FAK null, and FAK-E139A,D140A cells were plated on FN for 2 hr, wounded, and after 1.5 hr fixed and stained for the Golgi marker anti-GM130 (green), TRITC phalloidin (red), and DAPI (blue). Solid arrows show cells in which the Golgi is orientated toward the wound, and broken arrows indicate cells in which it is orientated away from the wound. Scale bars represent 20 μm. The percentage of each cell type with the Golgi orientated to the wound was calculated from a count of 100 cells in three experiments. Standard errors of means obtained in three individual experiments are shown.

(B) FAK-wt, FAK null, and FAK-E139A,D140A cells were seeded on transwell filters and allowed to invade Matrigel toward a serum gradient. After 72 hr cells were labeled with calcein AM and visualized by confocal microscopy in the Matrigel at 15 μm intervals. Quantification is shown in Figure S3B.

(C) FAK-wt and FAK-E139A,D140A cells were plated overnight, and random migration was then studied with an Olympus Scan-R timelapse imaging system. ImageJ was used for tracking the migration of 60 cells, and the pattern of migration (left panel) and the speed of migration were calculated (right panel). Cells that have changed direction are shown (arrows). Standard errors of means obtained in three individual experiments are shown.

case, 10% serum). After 72 hr, cells were labeled with calcein AM dye and visualized in the Matrigel at 15 μm intervals by confocal sectioning (Figure 3B), and the percentage of cells invading 90 μm into the 3D-gels was quantified (Figure S3B). FAK-deficient cells did not invade at all, and FAK-E139A, D140A-expressing cells displayed impaired chemotactic invasion relative to that of FAK-wt-expressing control cells (Figure 3B and Figure S3B). However, although slow, 2D planar migration of isolated tracked cells was unaffected by disruption of the FAK/RACK1 complex, and there was no difference in the ability of single cells to move or execute random turning manoeuvers in 2D culture (Figure 3C). These data imply that the FAK-E139A,D140A-expressing cells are impaired at sensing, and responding to, directional cues such as wounds or chemotactic stimuli, rather than at moving or turning, per se. The incomplete inhibition of invasion of SCC cells expressing FAK-E139A,D140A, and their delayed ability to repair a monolayer wound (Figure S3C), could be due to the fact that some cells are in the correct orientation for forward migration by chance and do not have to polarize, i.e., turn toward the serum gradient, to move into the Matrigel or the wound.

FAK and RACK1 Are in Complex with PDE4D5, which Is Required for Nascent Adhesions and Cell Polarization

In search of the mechanism by which the FAK/RACK1 complex mediates nascent adhesion assembly and directional responses, we considered known binding partners of RACK1. Cyclic AMP (cAMP) has been shown to affect cell shape and migration, and we knew that the cAMP-specific phosphodiesterase PDE4D5 interacts with RACK1 via its isoform-specific N-terminal region [16–18]. To examine whether PDE4D5 was associated with FAK in a complex that also contained RACK1, we immunoprecipitated FAK from FAK-wt- and FAK-E139A,D140A-expressing SCC cells and probed for the presence of both PDE4D5 and RACK1. Endogenous PDE4D5 associated with FAK in FAK-wt cells, but this association was reduced in cells expressing FAK-E139A,D140A (Figure 4A, quantified in Figure S4A). These data show that FAK is present in a larger complex together with RACK1 and PDE4D5 and that RACK1 probably acts as a molecular "bridge" linking FAK to PDE4D5. Consistent with this, the RACK1/PDE4D5 complex was independent of FAK's binding to RACK1 (Figure 4A, right panel). Importantly, there was a pool of endogenous PDE4D5 that colocalized with RACK1 at nascent protrusions at the substratum interface as cells were plated on FN, as shown by TIRF microscopy (Figure 4B). We also found colocalization of RACK1 and PDE4D5 in cells at the polarized edge of wounded monolayers (merged images in Figure S4B), and this was perturbed in FAK-E139A,D140A-expressing cells where neither RACK1

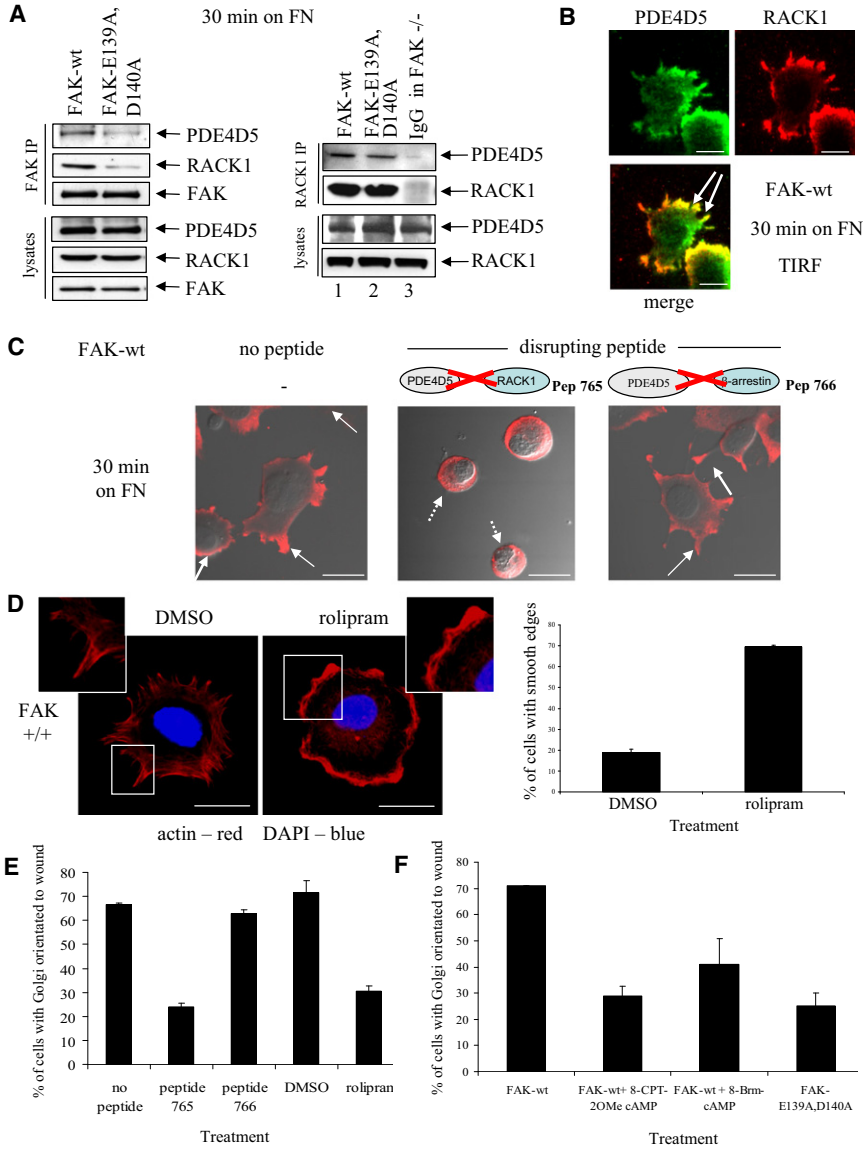

Figure 4. FAK and RACK1 Are in Complex with PDE4D5 and Colocalize at Nascent Adhesions

(A) FAK-wt and FAK-E139A,D140A cells were suspended, then plated on FN. Cells were immunoprecipitated with either anti-FAK agarose or anti-RACK1, and then immunoblotting was carried out with anti-PDE4D5, anti-RACK1, and anti-FAK as probes. Lane 3 shows a control IgG precipitation of FAK null cells.

(B) FAK-wt and FAK-E139A,D140A cells were plated on FN and then stained with anti-PDE4D5 (green) and anti-RACK (red). Adhesions at the cell substratum interface were detected with TIRF. A merged image is shown (colocalized RACK1 and PDE4D5 are yellow; solid arrow). Scale bars represent 20 μm.

(C) SCC cells were pre-incubated for 2 hr with 10 μM of peptide 765 (middle panel) or peptide 766 (right panel), which disrupted binding between PDE4D5 and RACK and between PDE4D5 and β-arrestin, respectively. Cells were plated on FN, then fixed and stained with anti-RACK1 antibody (red) and imaged by DIC. Solid arrows indicate the formation of protrusive structures, and broken arrows indicate rounded cells.

(D) Cells were suspended with 10 μM rolipram for 1 hr, plated on FN for 30 min in the presence of rolipram, and then fixed and stained with TRITC phalloidin and DAPI. Zoomed images and quantitation are also shown. Scale bars represent 20 μm. Cells with smooth edges rather than protrusive nascent adhesions are quantified.

(E) Polarization assays were carried out on SCC cells that were plated and wounded in the presence of peptide 765, peptide 766, DMSO, or rolipram. Cells were fixed and stained for the Golgi marker anti-GM130, TRITC phalloidin, and DAPI, and the percentage of cells with the Golgi orientated to the wound is shown. Standard errors of means obtained in three individual experiments are shown.

(F) Polarization assays were also carried out in the presence of 8-CPT-2OMe cAMP (10 μM) and 8-Brm-cAMP (10 μM). The percentage of cells with the Golgi orientated to the wound is shown. Standard errors of means obtained in three individual experiments are shown.

nor PDE4D5 efficiently localized to the polarizing edges (Figure S4B).

Next, we used a previously characterized peptide that, when stearoylated to make it membrane permeable, specifically disrupts the RACK1-PDE4D5 complex (peptide 765) in cells and compared its action with a peptide that specifically disrupts a PDE4D5 complex with another molecular scaffold protein, namely β-arrestin (peptide 766) [16, 17]). We examined cell spreading by DIC imaging of SCC cells in either the absence or presence of each of these stearoylated peptides (Figure 4D). There was only a striking loss of peripheral protrusions and spread morphology in cells incubated with the PDE4D5-RACK1-disrupting peptide 765 (quantified in Figure S4C). Importantly, we confirmed that, when compared to control peptide 766 (Figure S4D), peptide 765 suppressed binding to PDE4D5.

To further test the hypothesis that PDE4D5 has a role in mediating the effects we observed here, we made use of the PDE4-specific inhibitor rolipram (reviewed in [19]). Rolipram impaired the formation of protrusive nascent adhesions; the edges of treated cells had smooth edges with regions of dense cortical actin (the latter might have arisen from inhibition of PDE4D isoforms other than PDE4D5;Figure 4D). Furthermore, rolipram, and the RACK1/PDE4D5 disruptor peptide (peptide 765), inhibited polarization (Figure 4E), and rolipram inhibited chemotactic invasion (not shown). We also artificially elevated cAMP by use of the analogs 8-Brm-cAMP and 8-CPT-2-OMe-cAMP and found that these mimicked inhibition of polarization induced by RACK1-binding-impaired FAK mutant FAK-E139A,D140A (Figure 4F). Finally, we expressed a dominant-negative form of PDE4D5 generated by the mutation of Asp556 to alanine (described previously in [20]). Expression of PDE4D5-D556A was possible for short times and reduced formation of nascent protrusive adhesions when cells were plated on FN (Figure S4E, broken arrows). We confirmed that the dominant-negative PDE4D5 did not disrupt the RACK1/FAK complex (Figure S4E). Taken together, our data provide compelling evidence that the catalytic activity of PDE4D5 is required for generating and maintaining the spread phenotype and for stimulus-induced polarization. This is consistent with FAK/RACK1-recruited PDE4D5's functioning to keep cAMP levels low in a spatially constrained manner at nascent

adhesions as they form and at the leading edges of polarizing cells. We were not successful in knocking down RACK1 protein efficiently in the cancer cells used here, and we did not knock down PDE4D family PDEs because we reasoned that this would have been unlikely to phenocopy effects of the more subtle approaches we took to disrupt only one complex, namely the FAK/RACK1/PDE4D5 direction-sensing polarity complex.

Our data show that when the FAK-RACK1 complex is perturbed, neither RACK1 nor PDE4D5 can localize to nascent adhesion structures or to the leading edge of polarizing cells. The FAK/RACK1 complex serves to recruit PDE4D5 and promote spreading and polarization; RACK1 acts as an intermediary scaffold. The consequences of loss of the FAK/RACK1 complex mimic FAK deficiency and might contribute to FAK's role in promoting the invasive cancer cell phenotype that we have demonstrated previously in squamous cell and breast cancers in vivo [12, 21].

The mechanisms underlying assembly and distribution of signaling complexes as cells first contact the ECM remain unclear. Previous studies have identified protein complexes that regulate polarization in a number of contexts. For example, integrin-induced activation of Cdc42 controls the ability of astrocytes to polarize in response to a wound made in a confluent cell monolayer by leading to the activation and recruitment of the well-studied mPar6/PKCζ polarity complex [15, 22]. However, it is likely that other polarization-determining protein complexes, perhaps acting as part of a network, exist and that some of these modulate peripheral actin structures across cells to promote directionality.

Although cancer cells frequently lose apical-basolateral polarity, it is likely that polarization and directional migration toward chemotactic stimuli, such as cytokines or chemokines, play a role in metastatic spread during cancer invasion. Yet we do not understand how cancer cells turn toward polarization stimuli, and little is known about protein complexes specifically involved in direction sensing. We have found that FAK is needed for wound-induced polarization of SCC cells and chemotactic invasion through 3D matrix gels. In pursuing the mechanisms by which FAK functions, we have found that the complex between FAK and RACK1, and between RACK1 and its client protein PDE4D5, are required for SCC cancer cells to sense direction and respond. This complex is permissive, and essential, for assembly of actin-based protrusive structures at cell edges as cells spread or polarize in response to directional cues. Our time-lapse movies suggest that the FAK-RACK1 complex acts to stabilize a subset of actin adhesion structures as they form; in turn, this might specify cell shape (leading and trailing edges) and so directionality. We think we have uncovered one of the earliest spatially regulated signaling complexes that regulates nascent actin-based adhesion structures, the spread phenotype, and direction sensing in response to environmental cues.

Experimental Procedures

Cells

pWZL-FAK constructs were used for generating FAK-deficient (FAK-null) MEFs (control cells; kind gift from D. Ilic, StemLifeLine, CA), FAK-wt, FAK-Y397F, FAK-KD, and FAK-Y4F-Y9F reconstituted FAK-null SCC cells as pools with amaxa nucleofection kit v, program P20, and cells were maintained in 0.4 mg/ml hygromycin B.

In Vitro Complex Analysis

GST-RACK1 recombinant protein coupled to glutathione agarose beads (0.5 μg) was preincubated with either peptide 765 or 766 for 1 hr in PBS at 4°C prior to the addition of recombinant PDE4D5 (0.5 μg). The samples were incubated at 4°C for a further 30 min and washed three times in cold PBS, and SDS sample buffer was added. Samples were analyzed by immunoblotting with anti-PDE4D5 and anti-RACK1.

Immunocytochemistry and Cell Polarization Assays

Immunocytochemistry was performed as described in the Supplemental Information. For the analysis of cell spreading, cells were trypsinized, washed, and resuspended in PBS, then incubated with rotation at 4°C for 1 hr. Cells were then plated on FN-coated (0.01 mg/ml human FN) coverslips for the indicated times in serum-free medium. Cell-permeable peptides were added to the cells for 2 hr prior to rotation, and cAMP compounds were added prior to rotation and again upon plating. For polarization assays, cells were plated on FN at a density of 3×10^6 cells per well on a 12-well dish for 2 hr. Cells were then wounded with a pipette tip, washed twice in PBS, maintained in complete media for 1.5 hr, and then fixed. Cell-permeable peptides or cAMP compounds were added to the cells upon plating and again after wounding.

Invasion Assays

Invasion was assessed with an in vitro inverse invasion assay. In brief, 100 μl of growth-factor-reduced Matrigel was diluted 1:1 in cold PBS and allowed to set at 37°C for 1 hr in polycarbonate transwells. Cells (1×10^4) were seeded onto the underside of the transwell filter and allowed to adhere for 4 hr. Transwells were washed and placed in serum-free medium. Normal growth medium containing 10% serum was added above the Matrigel, and cells were allowed to invade for 72 hr. Cells were then stained with 5 μM calcein-AM for 1 hr at room temperature, and horizontal z sections through the Matrigel were examined at 15 μm intervals with an Olympus FV1000 confocal microscope. The amount of dye (positive pixels) was calculated for each z section with ImageJ software and expressed as a percentage of the section that represents the base of the transwell filter.

Random-Cell-Migration and Wound-Polarization Assay

For the random-migration and wound-polarization assays, 0.5×10^4 and 1×10^6 cells, respectively, were plated on a 6-well tissue culture dish in the presence of serum-containing media and allowed to adhere overnight. Plates were then washed with fresh media and placed in a microscope humidity chamber that was maintained at 37°C and supplemented with CO_2. Cell migration or wound closure was monitored for 15 hr with an Olympus scan R microscope, and sequential images were captured at 15 min intervals. Analysis was performed with imageJ software.

Supplemental Information

Supplemental Information includes three figures and two movies and can be found with this article online at doi:10.1016/j.cub.2010.04.042.

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